Insect vector transmission assays

Experimental phytoplasma transmissions by insects

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Summary

Phytoplasmas are transmitted in a persistent propagative manner by phloem-feeding vectors belonging to the order Hemiptera, suborder Homoptera. Following acquisition from the infected source plant, there is a latent period before the vector can transmit, so transmission assays consist of three basic steps: acquisition, latency and inoculation. More than ninety vector species (plant-, leafhoppers and psyllids) have been discovered so far but many others are still undiscovered, and their role in spreading economically important crop diseases is neglected. Therefore, screening for vectors is an essential step in developing rational control strategies for phytoplasma-associated diseases, targeted against the actual vectors. The mere detection of a phytoplasma in an insect does not imply that the insect is a vector; a transmission assay is required to provide conclusive evidence. Transmission experiments can be carried out using insects from phytoplasma-free laboratory colonies or field-collected. Moreover, transmission assays can be performed by feeding vectors on an artificial diet through Parafilm®, then phytoplasmas can be detected in the sucrose feeding medium by PCR. Transmission trials involve the use of different techniques according to the biology of the different vector species, planthoppers, leafhoppers and psyllids.

1. Introduction

Phytoplasmas are phloem-limited pathogens associated with a huge number of diseases in both cultivated and wild plants. In nature phytoplasmas are transmitted by insect vectors in a persistent, propagative manner. The insects acquire phytoplasmas feeding on an infected plant and then transmit them to a healthy plant after a latent period, during which the phytoplasma move through and multiplies in the vector body. The insects often remain infective for the entire lifespan.

Since phytoplasmas are phloem-limited their vectors, belonging to the order Hemiptera, are phloem feeders. Most of phytoplasma vectors are confined into three main taxonomic groups: leafhoppers (Auchenorrhyncha: Cicadellidae), planthoppers (Auchenorrhyncha: Fulgoromorpha) and psyllids (Sternorrhyncha: Psyllidae).

Even though phytoplasmas can be experimentally transmitted by grafting, by dodder (***1***), and in at least one case by root bridges (***2***), insect transmission is by far the most important manner of phytoplasma spread under field and natural conditions.

Seed transmission of phytoplasmas has been reported but, although phytoplasma DNA can be detected in embryos, there is as yet no evidence that the pathogen is seed transmitted through to the seedling to cause disease in progeny palms (***3***).

Vector insects can be polyphagous, oligophagous or strictly monophagous according to their ability to feed and reproduce on many, few or one host plant, respectively. Similarly, phytoplasmas may be generalists, infecting several different plant species, or specialists, infecting one or a few related

plant species. As a consequence, a generalist phytoplasma can be transmitted by several vector species. Plant-specialist phytoplasmas can be transmitted by a narrow range of vector species or by a specific vector (***4***).

When screening for phytoplasma vectors, PCR assays of field-collected insects may provide indications on the possible role of a given species in transmitting phytoplasmas. However, since phytoplasmas may be acquired but not injected with feeding (***5***), the mere detection of a phytoplasma in an insect does not imply that the insect is a vector; the transmission assay is the final evidence needed.

Transmission experiments are mandatory to i) identify new vectors ii) describe the transmission characteristics, such as transmission efficiency, duration of acquisition, incubation and inoculation periods iii) understand the epidemiology of a phytoplasma disease (is the vector feeding and breeding on the infected crop or is it a visiting insect that erratically transfer the phytoplasma from a weed or a natural reservoir into a crop species?). Even though nearly 100 phytoplasma vector species have been listed by Weintraub and Beanland (***6***), many of them are still undiscovered and their role in spreading economically important diseases is neglected.

The present chapter aims to provide the technical information necessary to perform transmission trials using leaf- and plant-hoppers as well as psyllids. First, the basic techniques for vector rearing are described, then methods for transmission experiments using laboratory-reared or field-collected insects are described. Finally, techniques used to assess phytoplasma transovarial transmission in the vector are presented. The different techniques are discussed for leaf- plant-hoppers and psyllids in the light of their different biology.

2 Transmission of phytoplasmas by insects

Phytoplasmas are transmitted by insect vectors in a persistent, propagative manner. Only selected species can act as vectors and the transmission of phytoplasmas by insects certainly involves, at several levels, elements of host-pathogen specificity (***4***).

Following acquisition from the infected source plant, there is a latent period before the vector can transmit, so transmission assays consist of three fundamental steps: acquisition, latency and inoculation.

During the acquisition phase the insect vector ingests phytoplasma particles feeding on an infected plant. Acquisition can be “natural”, when insects collected in the field, thus naturally infected, are used in transmission trials, or “controlled” when healthy putative vectors are caged on infected plants. In this latter case the period of time given to the insects to acquire the phytoplasma is called Acquisition Access Period (AAP). Usually AAP lasts from a few to several days to ensure a high acquisition efficiency. Even if few hours are enough for some species to acquire a phytoplasma (***7, 8***, author’s unpublished results), when the optimal AAP is unknown, long acquisition times should provide maximum efficiency.

The latency period (LP), also called “incubation period”, is the time interval between acquisition and the beginning of infectivity. During this phase the phytoplasmas invade the insect body *via* the haemolymph, multiply and reach the salivary glands. LP varies from 12 days to well over a month depending on the insect species, phytoplasma strain/species and abiotic factors such as

temperature (***5***). During this phase, the insects should be reared on a suitable plant to ensure the highest survival rate.
Once LP is completed, the insects inject the phytoplasmas directly into the sieve tubes of a healthy plant. Therefore putative vectors should be isolated, individually or in batches, onto healthy plants to assess phytoplasma transmission. The period of time given to the putative vector to transmit the phytoplasma is called Inoculation Access Period (IAP). Few hours can be sufficient to transmit the pathogen, but longer times can provide higher transmission efficiencies.
Based on the procedure, we can distinguish i) controlled transmission experiments (in which all the steps are carried out under controlled conditions in climatic chambers or greenhouses) ii) transmission experiments with field-collected insects (in which only the inoculation step is carried out under controlled conditions)

3 Controlled transmission experiments

Materials

- phytoplasma-free insect vector colony
- phytoplasma-infected source plants (source plants can be obtained by graft inoculation from diseased to healthy material, by a previous insect transmission, or by micropropagation of phytoplasma-diseased shoot cultures (***9***).
- potted seedlings of test plants
- climatic chambers or greenhouse or screenhouse
- rearing cages (plexiglas and/or net cages) (Fig. 1A e B)
- insect aspirator
- insecticides

Methods

- The availability of a laboratory colony of the vector depends mainly on the biology of the insect. Most of the leafhopper vectors (family Cicadellidae) are relatively easy to grow under controlled conditions since they breed continuously (multivoltine) or once a year (monovoltine) on one (monophagous), few (oligophagous), or many (polyphagous) host plants. Planthoppers are much more difficult to rear since nymphs are root feeder (***10***). In this case the rearing cage must include the soil with the roots of the host plants and attention must be paid to the disturbance or predation by other arthropods in the soil (e.g. ants) and to the watering of potted plants to avoid drowning of nymphs. The rearing technique has been described for the planthopper *Hyalesthes obsoletus* (***11***). Some psyllids (e.g. *Cacopsylla* spp.) are migrating insects that lay eggs and develop on a host plant and then migrate to shelter plants for aestivation and overwintering. In this latter case, even though, in theory, the complex cycle can be reproduced under controlled conditions, in the practice permanent rearings of these species are not feasible. In some

cases, e.g. leafhoppers laying eggs on woody hosts, it is possible to collect branches with eggs in the field, let them to hatch under controlled conditions, and establish an annual rearing. For *S. titanus*, the vector of Flavescence dorée, grapevine branches with eggs are collected in winter and stored in a fridge until needed (eggs are vital after some month storage); newly hatched nymphs feed on grapevine or broad bean plants and can be further used for transmission experiments. In our experience, broad bean plants are more suitable than grape, even though both plant species can be used (together or alternatively). As an option, *S. titanus* nymphs can be easily reared in small batches in cylindrical plastic boxes (10 cm height, 5 cm diameter), the top covered with a net and containing a 1-cm layer of technical agar solution (8%) on which a disk of grapevine leaf is laid and replaced twice a week (***12***). This system may avoid the use of plants when a limited number of individuals are needed.

- A laboratory colony of an insect vector can be established on a suitable host plant on which a multivoltine vector may breed several times per year. Basically we can describe two kind of continuous rearings: mixed-age rearing and age-structured rearing. In a mixed-age rearing a number of insects of all stages feed and breed on several plants. New plants are provided as soon as the old ones are ageing and dying and, when the insect population is too high, exceeding insects must be removed to ensure that plants can carry the population. When needed, nymphs are taken from the cage for transmission experiments. An age-structured rearing consists of an oviposition chamber where a number of females (together with males) lay eggs on host plants for a short period (from few days to one week). After the oviposition, host plants are moved to new cages where the eggs complete their embryonic development and give rise to the nymphs. Each cage contains plants exposed to ovipositing females at the same time and therefore all the nymphs are approximately of the same age. With this rearing, a lot of coetaneous nymphs can be conveniently used in transmission experiments. When dealing with monovoltine species with an obligate diapause (generally in the egg stage), continuous rearing is not feasible. For these latter the natural life cycle can be reproduced under controlled conditions or the insects can be obtained as described above for *S. titanus*

- Before starting the transmission experiments, a molecular analysis (PCR) should be done to ascertain the presence of phytoplasmas in the source plants

- A number of nymphs (preferably 3^{rd}-5^{th} instar nymphs) from the phytoplasma-free colony are caged onto source plants to feed for the AAP. The use of nymphs instead of adults is advisable since transmission of phytoplasmas requires that a relatively long latent period is completed in the insects before they can transmit. The use of adults will result in a high mortality before the latent period will be completed. On the other hand, 1^{st} and 2^{nd} instar nymphs are very small and delicate, so the manipulation of older nymphs is advisable. For Flavescence dorée phytoplasma (FD) it has been reported that

newly hatched nymphs fail to acquire the phytoplasmas from grape because their short stylets don't reach the phloem (***13***). AAP may last from one to several days according to the suitability of the source plant for the vector insect (if the source plant is a good host plant for the vector, longer AAPs result in higher acquisition efficiencies; if the source plant is a poor host for the insect longer AAPs result in high mortality and shorter AAPs are needed). AAP shorter than one day are not advisable since they can result in very low acquisition efficiency. AAP may take place in a greenhouse or in a climatic chamber and nymphs can be caged on the whole plant/s when using small potted plants as a source; alternatively AAP may take place in the field inside net cages isolating a single branch of the source plant

- Insects (nymphs and adults) surviving the AAP are transferred onto suitable host plants to complete latent period (LP)

- At the end of the LP insects should be caged on healthy test plants for an inoculation access period (IAP). Insects can be either caged in large groups on a number of test plants inside a plexiglas and nylon cage or a net cage, or caged singly or in small groups (e.g. 3-5 insects) on individual plants inside small cages. These can be glass or plexiglas cylinders, topped with insect-proof nets (Fig. 1C and D) or net cages isolating a single branch of a larger test plant (Fig. 1E). The duration of the IAP may vary and, as a general rule, longer IAPs result in higher transmission rates. As stated for AAP, if the test plant is a poor host for the insect, longer IAPs result in high mortality and shorter IAPs are needed to avoid high mortality. Nevertheless, if one wishes to maximize transmission efficiency, the vector insects can be maintained on the same test plant/s until death. Successive transfers of infectious insects on different test plants may allow to obtain a high number of infected plants, provided that the LP is completed. Few test plants should be exposed to healthy insects of the same species (from the lab-reared colony) as a negative control. These plants, representing the healthy control, will also allow a reliable and comparative evaluation of the symptoms expression by test plants exposed to infectious insects. To study phytoplasma movement in the plant, vectors can be caged on a restricted site of the foliage (e.g. apical or basal leaves). To achieve this, test plants can be covered with aluminium foil except for one or few apical or basal leaves (***14***). Alternatively, vectors can be caged on a single leaf using clip-cages (***15***) (Fig. 1F).

- In order to define the actual length of the LP under given environmental, after the AAP, the insects should be serially transferred, singly or in groups, to test plants for successive one or few day IAPs. The shorter the successive IAPs, the more precise is the estimation of the LP duration.

- At the end of the IAP, plants must be freed from insects, sprayed with insecticide and maintained in a greenhouse or climatic chamber to develop phytoplasma symptoms. If test plants are expected to be used as a source plants in new transmission experiments (e.g. for the

routine maintenance of a phytoplasma strain in the lab), non persistent insecticides (e.g. dichlorvos) must be applied or insecticide application should be avoided. Insects removed from test plants can be tested by PCR for phytoplasma presence, immediately or after storage in pure ethanol or at -20°C. In this way a correlation between acquisition and transmission efficiency can be obtained (number of phytoplasma-positive *vs* number of infective insects).

- After a variable time lapse from the inoculation, test plants should be analysed by PCR to check for the presence of phytoplasmas. When dealing with routine transmission of a phytoplasma on test plants that develop clear symptom of infection, detection of phytoplasmas is not needed. Test plants may become infected and develop symptoms at variable time post inoculation. Herbaceous hosts generally develop symptoms between 10 days and two months post inoculation and therefore should be checked by PCR at these time points, while for perennials, plants should be kept at least for one year post inoculation and then analysed by PCR. PCR assays are sensitive enough to reveal infection also before the symptoms are shown; in our experience, an aster yellows phytoplasma was detectable in daisy plants as soon as 4 days post inoculation while symptoms were manifested only from 12 days onwards (***14***).

4 Transmission experiments with field-collected insects

Materials

- sweeping net or beating tray
- potted seedlings of test plants
- climatic chambers or greenhouse or screenhouse
- rearing cages (plexiglas and/or net cages)
- insect aspirator
- insecticides

Methods

- a number of adults are collected in or around phytoplasma-infected fields using a sweeping net or a D-Vac equipment (plant and leafhoppers) or a beating tray (psyllids)
- field-collected insects are caged on test plants as described above. A long IAP is advisable (e.g. weeks or until insect death) since it is not known if infected insects from the field already completed the LP
- further steps in the transmission experiment are the same as described above

Transmission experiments with field-collected insects may provide evidence that the insect is actually a vector of the phytoplasma but do not provide evidence of the nature of the source plant and of the duration of the LP.

5 Transmissions to artificial feeding medium

Transmission experiments are generally performed by caging infectious insects onto susceptible test plants. However, since sucking insects can also feed through membranes, it can sometimes be useful to test vector ability on artificial media. This can be particularly useful when i) available test plants are poor hosts for the potential vector ii) a high number of insects, e.g. field collected insects, have to be tested for the infection rate under field conditions iii) screening for unknown vectors and therefore host plants are not known. In this latter case a lot of insects can be easily tested avoiding the production of a huge number of plants that must be maintained for a long period in greenhouse for disease development. Moreover, after vector feeding, the medium can be immediately analysed by PCR for phytoplasma detection and long incubation times in the plant are avoided. Actually, a number of phytoplasma transmission tests have been performed on artificial media (***16, 17, 18, 19, 20, 21, 4***) using plant- and leafhoppers. Even though only the successful transmission to the plant is the final evidence of the vector ability, transmissions to artificial feeding media may help in determining transmission capability. Due to the "artificial system", quantitative data from transmission experiments should be interpreted with caution since transmission capability/efficiency may be either overestimated (host plant may be partly tolerant and only some inoculation events may result in transmission while feeding media may contain phytoplasma cells injected with saliva that provide positive results in PCR assays) or underestimated (since the artificial feeding medium does not support phytoplasma multiplication, phytoplasmas are present in the diet in a low number, are rapidly degraded and feeding media may provide negative results in PCR assays).

Materials

- insect feeding media, e.g.
  a) 5% sucrose in TE (10 mM Tris, pH 8.0, 1 mM EDTA) (***18***)
  b) 10% sucrose, 0.2% fructose, 0.375% K_2HPO_4, 0.028% $MgCl_2$, pH 7.5
- microcentrifuge tubes or cup feeding chambers
- Parafilm® membrane

Methods

- The feeding chamber is prepared by
  i) filling the lid of a microcentrifuge tube with 200 µl of feeding medium and closing the lid with parafilm. The bottom of the tube is then cut, an insect is isolated inside the microcentrifuge tube, then closed with a small cotton wool ball (Fig. 1G)
  ii) stretching a first parafilm sheet on the top of a plastic cup, adding feeding medium on the parafilm and closing with a second parafilm sheet (we use a 45 mm diameter cups filled with 800-1000 µl of diet). Some leafhoppers are isolated inside the cup (we generally cage 5 leafhoppers per cup) through a hole, that is then closed with a small cotton wool ball (Fig. 1H). Tubes and cups should be maintained with the cap facing a light source to attract the insects to the feeding medium.

Besides feeding, leafhoppers also lay eggs through the Parafilm® into the feeding medium.

- At the end of the inoculation period, the feeding medium is collected with a pipette and a DNA extraction procedure is carried out that avoid the presence of the sugar in the template for PCR
- Phytoplasma cells are pelleted out of the feeding solution by centrifugation at 12,000 g for 15 min. Genomic DNA is extracted by adding 10 µl of 0.5 M NaOH, followed by the addition of 20 µl of 1M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate and 20mM EDTA. The mixture is then incubated at 65°C for 15 min, precipitated with 2 volumes of ethanol, redissolved in 30 µl of TE, and kept at -80°C (***18***). The amounts of reagents are detailed for 200 µl of artificial diet and should be adjusted accordingly when using higher amounts of diets (e.g. in the cups).
- 2 µl of the extract are used as a template in direct PCR, then followed by a nested PCR

Several different liquid diets of different complexity have been proposed for leafhoppers but, for most of the work with vector transmission of phytoplasmas a simple feeding solution, buffered sucrose, can be conveniently used (***22***). It is also possible to feed insect vectors on a solid diet, consisting of solidified acqueous solution of 5% sucrose and 4% low-melt agarose wrapped with Parafilm® (***23***), but then a protocol for the isolation of phytoplasmal DNA from this medium must be experienced.

For some sap-sucking insects, Teflon membranes with pores of about 1 µm have been used instead of Parafilm® with good results (***24***). So far, for phytoplasma vectors the use of these Teflon, hydrophobic membranes have not been reported, and preliminary results obtained in our laboratory seems to indicate that leafhoppers don't feed through these membranes. This is maybe due to the size of the pores, 1 µm, that are too small for the insertion of the stylets. Membranes with larger pores produce liquid diet leaking through the pores and therefore are not suitable.

6 Evaluation of transmission experiment results

Results of the transmission experiments are based on the evaluation of the symptoms developed by the test plants exposed to the inoculation by the vectors and/or by PCR assay of the test plants to detect the phytoplasmas. When dealing with transmission experiments through Parafilm® into the artificial diet, PCR assay (generally nested PCR to increase sensitivity) is obviously mandatory to achieve transmission results.

When transmission trials are carried out using groups of insects, the actual proportion of infectious insects can be estimated using the maximum likelihood estimator of p, $p\hat{} = 1 - Q^{1/k}$ where Q is the observed fraction of non-infected plants and k is the number of insects

per plant, assuming that the vectors acted independently (***25***). Obviously, the smaller the groups and more precise is the estimation. Group-transmission experiments allow to conveniently test a large number of field-collected vectors, without losing information on their infectivity rate.

7 Transovarial transmission experiments

The transovarial transmission has been proved for the leafhopper *Matsumuratettix hiroglyphicus*, vector of the sugarcane white leaf phytoplasma (***26***) and for *C. pruni* as a vector of "*Ca*. Phytoplasma prunorum" (***27***). For other phytoplasma-vector associations only the presence of phytoplasma cells or DNA in the progeny of leafhoppers fed on phytoplasma-infected plants has been proved (***28, 29***). Transovarial transmission trials can be carried out with either field collected or phytoplasma-free insect vector from laboratory colonies.

Materials

- sweeping net or beating tray
- potted seedlings of test plants
- phytoplasma-infected source plants
- climatic chambers or greenhouse or screenhouse
- rearing cages (plexiglas and/or net cages)
- insect aspirator
- insecticides

Methods

- for vectors from laboratory colonies, nymphs should be first caged onto source plants for the AAP and then maintained on the same or different plants until emergence

- one virgin female together with one or more male/s or individual mated females are isolated on potted seedlings of a phytoplasma-immune plant species for mating and/or oviposition. When dealing with a monophagous insect that can not be reared on an alternative immune host, infectious adults can be caged on twigs (Fig. 1I) of the susceptible plant species but, after the oviposition, twigs hosting the eggs are moved into cages with healthy potted seedlings to allow development of the nymphs, thus avoiding acquisition through the plant (***27***)

- after laying a sufficient number of eggs, the adults are removed and females tested by PCR for phytoplasma presence. Only the eggs laid by infected females are considered for further steps

- at different times after oviposition, some eggs, nymphs and newly emerged adults are sampled and tested by PCR for phytoplasmas

- the remaining adults are caged onto healthy potted seedlings

for transmission trials. At the end of the IAP all the adults should be tested for phytoplasma presence

- after a variable time lapse from the IAP, test plants should be observed for symptoms and analysed by PCR to check for the presence of phytoplasmas.

To assess the presence of phytoplasmas eggs, nymphs and newly emerged adults can be tested singly or in batches. For eggs, that may represent a poor DNA target, batches are more feasible or, alternatively, single eggs can be tested by PCR for both phytoplasma and an internal insect control gene, to avoid false negative results (***30***). The presence of the phytoplasmas in all the stages originating from the same infected female provides evidence of phytoplasma DNA inheritance to the progeny, while successful transmissions by the progeny adults is the final evidence of the vertical infectivity transmission.

8 Conclusions

This chapter describes the most common techniques used for phytoplasma transmission by vector insects. Since the mere detection of a phytoplasma in an insect does not imply that the insect is a vector, the transmission assay is required to provide conclusive evidence.

Basically, three different types of transmission experiments have been described: a) completely controlled transmissions, in which AAP, LP and IAP are carried out under controlled conditions b) transmissions with field-collected insects, in which only IAP is carried out under controlled conditions c) transmission to artificial diet through Parafilm®, in which test plant is replaced by a sucrose feeding medium. To perform fully controlled transmission experiments the establishment of a phytoplasma-free colony of the vector is mandatory.

Besides phytoplasma transmission experiments to plants, also transovarial transmission experiments to the progeny have been described.

Transmission experiments provide information on transmission capability and efficiency, duration of acquisition, latency and inoculation periods. Molecular detection of phytoplasmas in the source and test plants as well in the insects used in the transmission experiments is very useful and sometimes essential to achieve reliable results. Transmission trials involve the use of different techniques according to the biology of the different vector species, planthoppers, leafhoppers and psyllids. As a general rule, monoic, multivoltine, polyphagous, leaf feeding vectors are easier to manipulate in transmission experiments; unfortunately several vectors are migrating and/or monovoltine and/or monophagous and/or root-feeder insects and transmission experiments must be adapted accordingly to these characteristics.

For some phytoplasma species/strains, vectors have not been discovered yet and for many others it is likely that many more species than actually known act as a vector. For these reasons, many more transmission experiments are needed to identify new vectors, describe the epidemiology of phytoplasma

diseases and design rational control strategies.

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Fig. 1. (**a**, **b**) Plastic and net cages for vector rearing (**c**): net cage isolating a single branch for phytoplasma acquisition in the field (**d**, **e**): glass cylindrical cages for phytoplasma inoculation in the laboratory (**f**): net cage isolating a single branch of a test plant for phytoplasma inoculation in the laboratory (**g**): clip cage (**h**, **i**): microcentrifuge tube cage for phytoplasma inoculation to feeding medium with a single insect (**j**): cup-like cage for phytoplasma inoculation to feeding medium with insect groups (**k**): glass tube with an apple twig for egg laying in transovarial experiments.